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van de Vossenberg, J.L C M; Driessen, A.J.M.; da Costa, M.S.; Konings, W.N

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Homeostasis of the membrane proton permeability in *Bacillus subtilis* grown at different temperatures

Jack L.C.M. van de Vossenberg [a], Arnold J.M. Driessen [a], Milton S. da Costa [b], Wil N. Konings [a,*]

[a] *Department of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands*

[b] *Departamento de Bioquímica, University of Coimbra, Apartado 3126, 3000 Coimbra, Portugal*

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Abstract

Bacillus subtilis was grown at its growth temperature limits and at various temperatures in between the lower and upper growth temperature boundary. Liposomes were made of the extracted membrane lipids derived from these cells. The headgroup composition of the cytoplasmic membrane lipids did not differ significantly at the lower (13°C) and upper (50°C) temperature boundary. The averaged lipid acyl chain length, degree of saturation, and ratio of iso- and anteiso-branched fatty acids increased with the temperature. At the temperature of growth, the membranes were in a liquid–crystalline phase, but liposomes derived from cells grown at 13°C were almost threefold more viscous than those derived from 50°C grown cells. The temperature dependence of the proton permeability of the liposomes was determined using the acid-pulse method with monitoring of the outside pH with the fluorescent probe pyranine. The proton permeability of each liposome preparation increased with the temperature. However, the proton permeability of the liposomes at the growth temperature of the cells from which the lipids were derived was almost constant. These data indicate that the growth temperature dependent variation in lipid acyl chain composition permits maintenance of the proton permeability of the cytoplasmic membrane. This 'homeo-proton permeability adaptation' precludes futile cycling of protons at higher growth temperatures and allows cells to sustain the proton motive force as a driving force for essential energy transducing processes.

Keywords: Proton permeability; Temperature dependence; Homeo-proton permeability adaptation; (*Bacillus subtilis*)

1. Introduction

All organisms have a specific temperature range in which they can grow. For most bacteria the difference between lower and upper growth temperature is around 30°C [1]. They respond to changes in ambient temperature through the adaptation of the lipid composition of the cytoplasmic membrane [2]. In *Escherichia coli*, this adaptation mechanism results in a constant membrane fluidity at different growth tem-

Abbreviations: DSC, differential scanning calorimetry; *k*, first-order rate constant for proton permeability; TMA-DPH, 1-[4-(trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene; CL, cardiolipin; PG, phosphatidylglycerol; PE, phosphatidylethanolamine

* Corresponding author. Fax: +31-50-363-2154; E-mail: w.n.konings@biol.rug.nl

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peratures in a process that is termed 'homeoviscous adaptation' by Sinensky [3]. However, in *Bacillus subtilis* [4] and many other bacteria [5–7], the membrane fluidity at the growth temperature was found to increase linearly with the growth temperature, and thus does not remain constant within the growth temperature range. That urged Sinensky to modify the original homeoviscous adaptation concept into 'homeoviscous efficacy' [8], describing the extent of homeoviscous adaptation. Growth temperature dependent changes of the lipid composition of the cytoplasmic membranes of bacteria are mainly found in the fatty acyl chain composition of the membrane lipids, while the composition of the polar headgroups is less affected [9–11]. At higher growth temperatures, usually the number of unsaturated acyl chains declines, while the average length of the fatty acid chains, i.e., carbon number, increases.

Protons, and sodium ions, are the most commonly used coupling ions in energy transduction in bacteria and archaea. In a previous study we have investigated the influence of growth temperature on the proton permeability of liposomes prepared from membrane lipids derived from different organisms [12]. For mesophilic bacteria and archaea the first-order rate constant of proton permeation, which is proportional to the diffusion constant, was maintained at a constant value at the respective growth temperature. These data suggest that the proton permeability of the membrane is an important parameter for viability.

These observations led us to propose the homeoproton permeability adaptation theory that proposes that bacteria adjust their membrane lipid composition in such a way that the proton permeability of the membrane is maintained at a low level at the growth temperature. To test this theory, the influence of the growth temperature was studied on the proton permeability of liposomes prepared from lipids of *B. subtilis* grown at and between the boundaries of its growth temperature range. The results demonstrate that the growth temperature dependent alterations in fatty acyl chain composition do not only maintain the membrane in a liquid–crystalline state. More importantly, the alterations permit the cells to maintain a low proton permeability of the membrane within a wide range of growth temperatures.

2. Materials and methods

2.1. Strains and growth conditions

Bacillus subtilis DB104 (*Δapr-684, ΔnprE522*) [13] was grown on Luria broth at several temperatures between 13 and 50°C, with constant high aeration. Pre-cultures were grown at 25°C. At late logarithmic phase, i.e., $OD_{660\,nm}$ 1.7 to 2.5, protein synthesis was blocked by the addition of 5 μg/ml chloroamphenicol, and cells were harvested by centrifugation. Cells were resuspended in 50 mM potassium phosphate (pH 7), frozen as small nuggets in liquid nitrogen and stored at −80°C. Cells that were grown at 13°C were still able to grow at 50°C, and vice versa, indicating that growth was not due to mutation.

2.2. Lipid extraction and analysis

Crude lipids were chloroform/methanol extracted [14], and acetone-ether-washed [15]. For the 13°C and 16°C grown cells, the ether wash was conducted at 4°C. Lipids were stored in chloroform at −20°C under a N_2 atmosphere.

The acyl chains were analysed as methyl esters by gas–liquid chromatography [16]. The lipid headgroup composition was determined by two-dimensional thin-layer chromatography [17]. Chromatograms were stained with I_2 vapour, and spots were collected and analysed for inorganic phosphorous [18]. Data were derived from two independent batches.

2.3. Preparation of liposomes

Unilamellar liposomes with an averaged diameter of 200 nm were prepared by extrusion [12,19] in 50 mM MOPS, pH 7.0, 75 mM KCl, and 25 mM choline-Cl to a final concentration of 20 mg/ml.

2.4. Time-resolved and anisotropy fluorescence spectroscopy

Lifetime and anisotropy measurements were performed with an SLM-Aminco 4800C fluorimeter (SLM-Aminco, Urbana, USA) using the fluorescent probe TMA-DPH (1-[4-(trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene) [20]. Excitation and

emission were at 360 nm and 430 nm, respectively. The temperature dependence of the fluorescence polarization was determined using Glan–Thompson polarizers in the excitation and emission channels. Anisotropy values, r, were derived from the polarization as described [21].

The excited-state lifetime, τ, of TMA-DPH was determined by the phase delay technique at 18 MHz with excitation and emission bandwidth of 1 and 4 nm, respectively. The phase lifetime, τ_ϕ, was referenced against a glycogen scatter reference ($\tau = 0$ ns). The relation between τ_ϕ and the temperature was fitted by iteration to the best fitting linear function (TableCurve, Jandel Scientific, San Rafael, USA). For each temperature, the rotational correlation time, φ, was calculated from the anisotropy (r) and lifetime (τ_ϕ) according to Eq. 1:

$$\varphi = \frac{\tau_\phi}{(r_o/r)-1)} \quad (1)$$

in which the zero-time anisotropy r_o was fixed to the value 0.378 [22]. From the rotational correlation time, the viscosity, η, was calculated according to Eq. 2:

$$\eta = \frac{RT\varphi}{V} \quad (2)$$

in which R is the gas constant, T the absolute temperature (in K), and V the total enclosed volume of one mole of TMA-DPH. A value of 959 Å^3 was calculated form the solvent accessible surface of TMA-DPH using the QUANTA96 software package (Molecular Simulations, San Diego, USA) after energy minimalization with the CHARMm module.

2.5. Differential scanning calorimetry

The phase transition in the lipids of *B. subtilis*, grown at 13°C and 50°C, was measured by differential scanning calorimetry (DSC) using the Microcal MC-2 calorimeter (Microcal, Amherst, USA). Data were analysed using the ORIGIN software (Microcal Software), which involved fitting and subtraction of the instrumental base line data as described [23].

2.6. Proton permeability

The proton permeability of the liposomes was measured as described [12,19,24]. In short, liposomes (1.5 mg of lipid/ml) were transferred to a buffer containing 0.5 mM MOPS (pH 7.0), 75 mM KCl, 75 mM sucrose, and 10 μM of the fluorescent pH probe pyranine. The K^+ ionophore valinomycin (1 nmol/mg of lipid) was added to prevent the formation of a reversed $\Delta\psi$, and after equilibration and stabilization of the signal, 100 nmol H^+ (from a 50 mM H_2SO_4 stock solution) was added to the suspension to lower the external pH. Influx of protons into the liposomes was followed in time from the partial recovery of the external pH monitored by an increase in pyranine fluorescence (excitation and emission wavelengths of 450 nm and 508 nm, respectively). After calibration of the signal in the presence of nigericin, the data were fitted to the first-order kinetic equation as described [19]. The first-order rate constant of proton influx, k, was used for comparison of the proton permeability of the different liposomes. Fluorescence measurements were performed on a Perkin–Elmer LS-50B (Norwalk, USA) fluorimeter, using a thermostated, magnetically stirred sample compartment.

3. Results

3.1. Phospholipid headgroup and acyl chain composition

B. subtilis was grown at various temperatures between its lower and upper growth temperature boundaries, 13°C and 50°C. The doubling time during

Table 1
Influence of the growth temperature on the phospholipid headgroup composition of *B. subtilis* membranes

Headgroup	% Phospholipid phosphorous at growth temperature:	
	13°C	50°C
Phosphatidylglycerol and cardiolipin	54	53
Phosphatidylethanolamine	38	33
Lyso-phosphatidylethanolamine	n.d.	2.7
Phosphatidic acid	2.8	6.1
Unidentified	5	4.7

n.d., Not detectable.

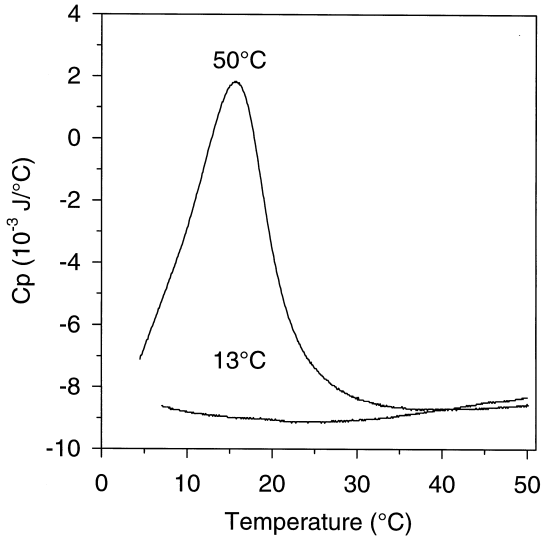

Fig. 1. DSC thermogram of liposomes prepared from lipids derived from *B. subtilis* cells grown at 13°C and 50°C. The scan rate was 0.5 and 1°C/min. Liposomes were used at 6 mg/ml in 100 mM potassium phosphate, pH 7.0. The reproducibility of the thermograms and reversibility of the transitions were checked after each run by re-heating the sample after cooling.

exponential growth was 18 h at 13°C, 6 h at 16°C and less than 1 h at the other temperatures. Phospholipids were isolated from cells grown up to the late exponential phase. The lipids from the cells, grown at the highest and lowest temperature, were used for further analysis. The phospholipids of cells grown at these two temperatures had similar headgroups (Table 1). Most of the lipids were cardiolipin (CL), phosphatidylglycerol (PG), and phosphatidylethanolamine (PE). The amount of phosphatidic acid (6.4%) at 50°C was about twice as high as at 13°C (4%). At 50°C, an increased content of *lyso*-phosphatidylethanolamine was measured, likely the result of some phospholipase activity during extraction. The growth temperature had a dramatic effect on the acyl chain composition of the phospholipids (Table 2). At 50°C, unsaturated acyl chains were completely absent. However, essentially all acyl chains were branched with twice as many iso-branched as anteiso-branched chains. The average fatty acyl chain length of the lipids of 50°C grown cells was slightly longer than in the 13°C grown cells. Cells grown at 13°C contained 9.6% unsaturated lipids, and had a 12-fold higher content of anteiso- than iso-branched lipids.

3.2. Phase transition and anisotropic behaviour of *B. subtilis* lipids

The phase transition behaviour of the lipids derived from cells grown at 13°C and 50°C was measured by differential scanning calorimetry (DSC) in liposomes in aqueous suspension. Lipids derived from cells grown at 50°C showed a discrete phase transition at 16°C (± 0.5, $n = 3$). The lipids derived from 13°C grown cells showed no phase transition between 5°C and 50°C. The phase transition of these lipids most likely occurs at a temperature below 5°C (Fig. 1).

The viscosity of the membranes was determined by TMA-DPH fluorescence anisotropy (r) and lifetime (τ_ϕ) measurements (Fig. 2). The temperature dependency of τ_ϕ for the TMA-DPH fluorescence with both the liposomes prepared from lipids derived from 13°C and 50°C grown cells is shown in Fig. 2 (upper panel). The longest lifetime component, which ac-

Table 2
Influence of the growth temperature on the fatty acyl chain composition of *B. subtilis* membranes

Acyl chain	% Of total at growth temperature:	
	13°C	50°C
13:0-iso	n.d.	0.5
14:0-anteiso	n.d.	1.2
15:0-iso	8.7	28.3
15:0-anteiso	50.3	21.6
16:1 ω7*c*	0.7	n.d.
16:0-iso	1.4	3.2
16:1 ω11*c*	0.6	n.d.
16:0	1	2.8
17:1 ω10*c*-iso	1.2	n.d.
17:1 ω10*c*-anteiso	7.1	n.d.
17:0-iso	2.7	29.9
17:0-anteiso	25.6	8.9
18:0	n.d.	0.6
19:0-iso	n.d.	2.2
19:0-anteiso	n.d.	0.8
Unidentified	0.8	n.d.
Ratio iso:anteiso	0.17	2.0
Unsaturation, % of total	9.6	0
Averaged acyl chain carbon number	15.75	15.93

The main changes upon increase in temperature are the transitions from anteiso-branched to the corresponding iso-branched lipids. n.d., not detectable. The standard error of the mean for each fatty acid was 4% ($n = 2$).

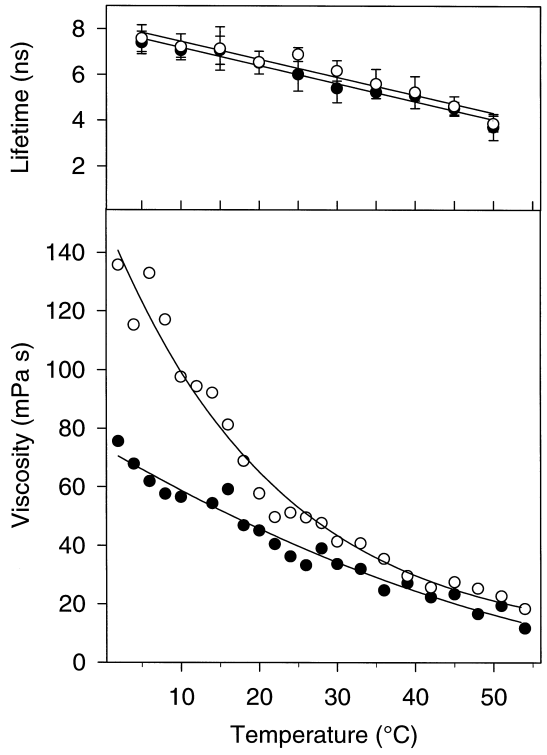

Fig. 2. Temperature dependency of the TMA-DPH fluorescence lifetime (upper panel) and apparent membrane microviscosity (lower panel) for liposomes prepared from lipids derived from *B. subtilis* cells grown at 13°C (●) and 50°C (○). Membrane microviscosity was calculated from the TMA-DPH fluorescence anisotropy and lifetime as described in Section 2.

counts for more than 85% of the fluorescent signal [22], decreased from about 7.7 ns at 5°C to 3.9 ns at 50°C. By combining the anisotropy data (not shown) with the lifetimes, the apparent viscosity, η, of the membranes as a function of the temperature was calculated (Fig. 2, lower panel). At the respective growth temperatures, η is about 55 and 15 mPa s for cells grown at 13°C and 50°C, respectively. The first derivative of anisotropy data of 50°C grown cells indicated a phase transition at 15°C, close to the value determined by DSC. Liposomes prepared from the lipids of 13°C grown cells showed no distinct transition.

3.3. Proton permeability of the liposomes

The temperature dependence of the proton permeability of the liposomes derived from 13°C and 50°C was determined by the acid-pulse method. From the proton influx data ($n \geq 8$), the first-order rate constants for proton permeability, k, were calculated and plotted versus the temperature (Fig. 3). The activation energy, derived from the slope of the log fit of k to the temperature, was comparable for these liposome preparations, yielding an averaged value of 78 ± 14 kJ/mol. These data demonstrate that the increase in proton permeability with temperature is similar for the two liposome preparations tested. However, at the higher growth temperature, the line shifted to a higher temperature range suggesting the maintenance of the proton permeability at the respective growth temperature. To analyse this phenomenon in further detail, the proton permeability was determined of liposomes made of lipids derived from cells grown at different temperatures between 13°C and 50°C. Permeability measurements ($n \geq 3$) were performed only at the growth temperature of the cells from which the lipids were derived. Above 40°C, reliable measurements of the proton permeability were not possible, due to the low amplitude of the measured signal. To overcome this problem, we determined the rate constant of the exponential fits in Fig. 3 at the growth temperature. As shown in Fig. 4, the proton permeability of the different liposomes was essentially constant at the temperature that corresponds with the growth temperature of the cells from which the lipids were extracted. These data

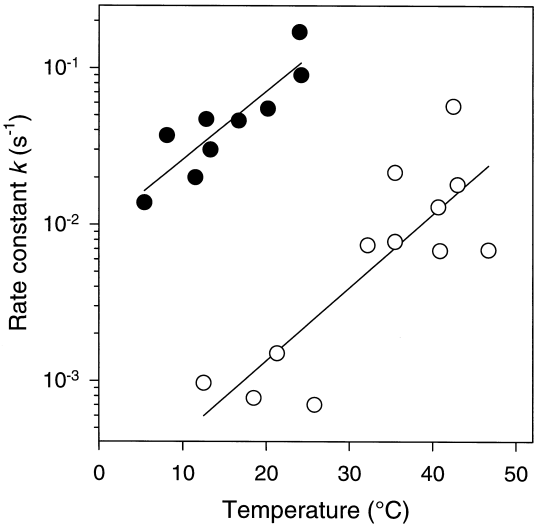

Fig. 3. Temperature dependency of the proton permeability of liposomes prepared from lipids derived from *B. subtilis* cells grown at 13°C (●) and 50°C (○). The influx of protons, as measured with the fluorescent probe pyranine, was fitted to a first-order kinetic equation as described in Section 2.

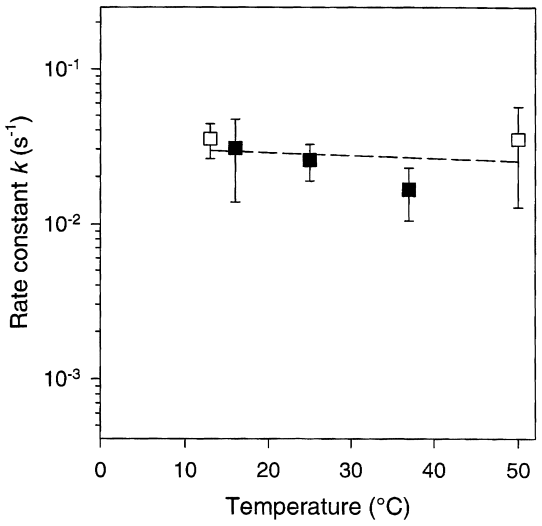

Fig. 4. Proton permeability at the growth temperature of liposomes prepared from lipids derived from *B. subtilis* cells grown at temperatures between the lower and upper temperature limit (■ ± S.D., $n \geq 3$). Inter- and extrapolation of the exponential fit on growth temperature curves at 13°C and 50°C, shown in Fig. 3 (□ ± 95% confidence intervals). The influx of protons, as measured with the fluorescent probe pyranine, was fitted to a first-order kinetic equation as described in Section 2.

indicate that *B. subtilis* cells maintain a constant proton permeability of their membrane at various growth temperatures.

4. Discussion

The bacterial membrane plays a crucial role in energy transduction of the cells. One of its essential functions is to preserve the barrier function to protons (or sodium ions) in order to keep the proton motive force (or sodium motive force) at a high value [25]. Bacteria therefore must be able to control the permeability of the membrane for these energy transducing ions. So far, the proton permeability of biological membranes has hardly been studied in relation to the growth conditions or physiology of the organism.

We have previously shown that the proton permeabilities – at the growth temperature – of the membranes from different psychrophilic or mesophilic bacteria and mesophilic or thermophilic archaea are comparable, even when the growth temperatures of the organisms studied are ranging from 4°C to 85°C [12,19]. The range of growth temperatures in that study was even wider than in the present study. The previous study suggested that bacteria and archaea possess mechanisms to adjust the membrane lipid composition at different growth temperatures in such a way that the proton permeability of their membranes remains constant. To test this 'homeo-proton permeability' theory we have studied the proton permeability of liposomes prepared from lipids of cytoplasmic membranes of *B. subtilis* grown at temperatures between 13°C and 50°C. The result of this study indeed shows that the temperature-dependent changes of the lipid composition in *B. subtilis* result in a membrane with a nearly constant proton permeability, irrespective of the growth temperature.

If bacteria would not be able to adapt the proton permeability of their membrane, the temperature range of growth of bacteria would be narrow. Without an adaptive mechanism, the proton permeability of the membrane would increase rapidly as in Fig. 3 [1], and cells would not be able to maintain a proton motive force. Our current study shows that a bacterial cell is able to maintain the proton permeability of the cytoplasmic membrane at a low value within a range of growth temperatures. The change in lipid composition with increasing growth temperature clearly counteracts the temperature effect on the proton permeability.

The permeability of the membrane depends on physico-chemical characteristics such as the lipid packing. In this respect, Lande et al. [26] observed that the permeability of small solutes in synthetic liposomal membranes correlates exponentially with the membrane fluidity, while an apparent relationship between fluidity and permeation of protons was not observed. Possible mechanisms for proton permeation across the membrane are summarized by Marrink et al. [27], who studied the molecular dynamics of the formation of water chains across the membrane. Those studies indicate that proton permeation may occur via a transient water wire through the membrane.

The growth temperature results in major changes in acyl chain composition of the membrane lipids of *B. subtilis* [6,11,16,28–30]. The most significant effect is a drastic decrease in the anteiso-branched fatty acids and an increase in the iso-branched fatty acids with increasing growth temperature (Table 2). These

variations in the lipid composition allow the cytoplasmic membrane to remain in a liquid–crystalline state at different growth temperatures [5,31,32]. The phase transition temperature is kept below the growth temperature due to the presence of branched lipids that distort membrane order. Anteiso-branches lower the transition temperature better than iso-branches, though neither are as efficient as the presence of a *cis* double bond in the centre of the hydrocarbon chain [28]. The efficient *cis* double bonds are observed in the cells grown at 13°C.

It has been proposed that bacteria need to maintain a constant viscosity of their cytoplasmic membrane at different growth temperatures. This 'homeoviscous adaptation' [3,33,34] has been postulated as the maintenance of the membrane fluidity to allow membrane proteins to function optimally. In *B. subtilis* and other organisms, homeoviscous adaptation is not as strict as in *E. coli* (see introduction and Fig. 2).

Shaw and Ingraham [10] found that the minimum growth temperature of *E. coli* is not governed by the rigidity of lipids in the membrane. When cells are shifted from 37°C to 10°C, a major lag phase occurs before the cells start to grow. Starvation during this lag period did not affect the duration of the lag phase, and no changes in fatty acyl chain composition were observed. However, when the cells were supplemented with glucose, just after the lag phase, growth immediately started at 10°C while the fatty acyl chain composition was still typical for cells grown at 37°C. The reversed experiment has not been done. Taken together with the observation that the fluidity is not so strictly maintained in *B. subtilis*, we may conclude that maintenance of the proton permeability characteristics of the membrane is even more important for viability of the cells than homeostasis of the viscosity.

It has been suggested that the changes in lipid composition are needed to maintain an optimal lateral pressure in the membranes at different temperatures [22]. This pressure is caused by phospholipids such as PE, which can form non-bilayer configurations, and depends on the type of acyl chain as the wedged shape of PE increases with the degree of unsaturation of the acyl chains. In the thermophilic bacterium *B. caldotenax*, lowering of the growth temperature results in a decreased PE content, which is compensated by an increased amount of unsaturated acyl chains [35]. However, the drop in PE content is far less dramatic than the change in acyl chain composition. In the present study and in *E. coli* [33], no change in PE content is observed as a function of the growth temperature. This suggests, that for adaptation of the membrane to higher temperatures the acyl chain composition is more important than the headgroup composition.

The ability of the cell to control the permeability characteristics of the membrane may be important in determining the upper temperature limit of growth. To some extent, an increased proton permeability of the membrane at higher temperatures could be compensated by an elevated rate of proton extrusion [36]. However, without any adaptation of the membrane lipid composition upon higher temperatures, the proton permeability will become too high upon small increases of the temperature and maintenance of a viable proton motive force and homeostasis of the intracellular pH will not be possible. This study therefore adds a new dimension to the role of the growth-dependent alterations in the lipid composition of cytoplasmic membranes. For future research, it will be important to elucidate the regulatory mechanisms responsible for this homeo-proton permeability adaptation of the cytoplasmic membrane.

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